Selenium Enriched *Bacillus Subtilis Yb-1*114246 Improved Growth and Immunity of Broiler Chickens Through Modified Ileal Bacterial Composition

Jiajun Yang (yjj1984112@outlook.com)
School of Animal Husbandry and Veterinary Medicine, Jiangsu Vocational College of Agriculture and Forestry
Jiang Wang
School of Animal Husbandry and Veterinary Medicine, Jiangsu Vocational College of Agriculture and Forestry
Kehe Huang
Nanjing Agricultural University College of Veterinary Medicine
Qingxin Liu
School of Animal Husbandry and Veterinary Medicine, Jiangsu Vocational College of Agricultrue and Forestry
Xiaozhou Xu
School of Animal Husbandry and Veterinary Medicine, Jiangsu Vocational College of Agriculture and Forestry
Guofang Liu
School of Animal Husbandry and Veterinary Medicine, Jiangsu Vocational College of Agriculture and Forestry
Hao Zhang
College of Animal Science and Technology, Chinese Agricultural University
Mengling Zhu
School of Animal Husbandry and Veterinary Medicine, Jiangsu Vocational College of Agriculture and Forestry

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Abstract

Background: Selenium-enriched *Bacillus subtilis* (SEBS) was made to add into the diets of broiler chickens to observe combined effects on the bacterial composition and immunity of ileum through its colonization on the site of intestinal mucous membrane to improve the body growth and immunity. Five hundred 1-d-old chickens were divided into five groups randomly: Control, inorganic Se, *Bacillus subtilis* (*B. subtilis*), SEBS, and antibiotic. The feed duration was 42 days (d). Growth performance was recorded and calculated. The strain of *B. subtilis* and SEBS colonizing in the gastrointestinal tract (GIT) were investigated using fluorescence in situ hybridization (FISH) assay and quantitative real-time polymerase chain reaction (qPCR). The V3-V4 hypervariable regions of the bacterial region of 16S rRNA gene were sequenced.

Results: After 42 d of treatment, chicks feed SEBS or *Bacillus subtilis* had higher body weights than the control chicks or those given inorganic Se. SEBS colonized in distal segments of the ileum improved bacterial diversity, while reducing the number of endogenous pathogen and increasing the number of *Lactobacillus* sp. in ileal mucous membranes. Species of unclassified *Lachnospiraceae*, uncultured *Anaerosporobacter*, *Peptococcus*, *Lactobacillus salivarius*, and *Ruminococcaceae_UCG-014*, and unclassified *Butyricicoccus* in ileal mucous membranes played an important role in promoting immunity, such as tumor necrosis factor-α and interferon-β through spearman's analysis. Dietary supplementary Se also improved bacterial composition of ileal mucous membranes. SEBS colonization in the ileal mucous membrane optimized bacterial composition, more enhanced the abundances of metabolic and immune genes on body growth and immunity.

Conclusions: SEBS improved body growth performance and immunity of broiler chickens through colonization in the ileal mucous membrane and modified ileal bacterial composition.

1. Introduction

Probiotics contain live microorganisms and spores and confer health benefits to the host when administered in adequate amounts [1]. Certain probiotic *B. subtilis* strains administrated orally survived, colonized the intestinal mucous membrane, and optimized the composition of microbiota, positively stimulating the intestinal immunity and metabolism [2]. These benefits helped in overcoming infection stress and clearing pathogens [3, 4].

In our previous research, the strain of *B. subtilis yb-1*14,246 was isolated from the ileum of chick, in which its probiotic effects have been proven in use on the growth performance of the chickens [5]. The same probiotic-enhancing characteristics of *B. subtilis yb-1*14,246 was also detected in the production performance of old laying hens using the supplementation [6]. One of potential characters of improving the growth was that *B. subtilis yb-1*14,246 can secret the digestive enzymes namely protease, lipase, and amylase to digest the digesta in intestine [6]. The colonization of probiotic bacteria in gastrointestinal tract (GIT) is important factor to play its role in interplaying with host [7, 8]. Once the probiotic bacteria can colonize in the site of intestinal mucous membrane. They utilized the nutrition in GIT to propagate themselves and secret the digestive enzymes to help the body digestion [3, 9]. This interaction was stronger than no colonized action of probiotic bacteria. Therefore, evaluation the colonization of *B. subtilis yb-1*14,246 is essential to make sure its approach of action [10]. Thereafter, to ensure the colonization of *B. subtilis yb-1*14,246 on the mucous membrane of ileum, further changed the ileal mucous bacterial composition and led to improved innate immunity and body growth is urgent.

Selenium (Se) is an important trace element and its beneficial effects are well-documented [11]. Suitable doses of Se supplementation can regulate metabolism and antioxidation [12]. Further, Se can help immune cells to defend against infection-causing pathogens [13, 14]. It also reported that supplemental se can modulate the bacterial composition in GIT, induced to good body health [15, 16].

Considering the beneficial effects of Se and *B. subtilis yb-1*14,246 on body [17, 18], it would be valuable to study Se and *B. subtilis yb-1*14,246 in combination to determine whether greater effects are induced when administered together. To study this, we produced Se-enriched *B. subtilis* (SEBS), which combines the benefits of *B. subtilis yb-1*14,246 and those of organic Se. This study discovered that SEBS colonizing the ileal mucous membrane while the whole immune status of the intestine was improved with the combined use of Se and *B. subtilis*, improved the anti-infection ability. This study could provide novel insights into the combined use of probiotic bacteria and the essential micro-element Se.

2. Materials And Methods

2.1. Preparation and analyses of BS and SEBS

*B. subtilis yb-1*14,246 (BS) was isolated from the ileum of a healthy Chinese Huainan Partridge chicken by our research group, the institute of animal husbandry and veterinary medicine, Anhui Academy of Agricultural Sciences, and stored at the China General Microbiological Culture Collection Center (CGMCC); the strain number is CGMCC 14246. The 16S ribosomal DNA was sequenced and deposited at the National Center for Biotechnology Information (NCBI) of the United States of America (USA) under the access number KT260179. We cultured *B. subtilis yb-1*14,246 using a liquid beef extract peptone medium. The fermentation of selenium-enriched *B. subtilis yb-1*14,246 (SEBS) was performed with sodium selenite supplemented into the culture medium. The morphological and structural properties of *B. subtilis yb-1*14,246 and SEBS were monitored using a scanning electron microscope (SEM) and a transmission electron microscope (TEM). *B. subtilis yb-1*14,246 and SEBS were concentrated via centrifugation at 3, 000 rpm, and immersed in a 5% glutaraldehyde solution for 24 h [19]. Se concentration in the supernatant and precipitate of *B. subtilis yb-1*14,246 and SEBS fermented medium was calculated using atomic absorption spectrometry and the live bacteria were enumerated with yeast extract peptone dextrose medium after term serial dilution.

2.2. Chicks and management

Animal experiments were approved and performed in accordance with the experimental guidelines of the Institutional Animal Care and Use Committee of China. The experimental protocols in this study, including animal husbandry and slaughter, were approved by the Institute of Animal Science and Welfare of Anhui Province (no. IASWAP2017120649). A total of 500 one-day-old Cobb broilers (average body weight, 40.05 g) were randomly allocated to five groups with five replicates of 20 each. The chickens were allowed *ad libitum* access to water and feed throughout the experimental period. The normal immune procedure was implemented throughout the trial.

2.3. Feed for each group

Chickens in the control group were fed a basal diet and the four treatment groups were fed the following: basal diet with either inorganic sodium selenite (IS), *B. subtilis yb-*114,246 (BS), Se-enriched *B. subtilis yb-*114,246 (SEBS), and flavomycin. Experimental diets were fed in two periods: starter (days 0–21) and finisher (days 22–42). The basal diet composition, which did not include any probiotics or antibiotics, and nutrient analysis results, are shown in Table 1. All nutrients met or exceeded the nutrient requirements of national research council (NRC, 2012) [20]. For the diet for chickens in the IS group, 1.12 g of sodium selenite (analytically pure) was diluted in 100 mL distilled water and blended with 5 kg of feed. Thereafter, the mixed mass feed was added to a blender containing 90 kg of mass feed. The blender was employed for 20 min to ensure uniform mixing of additives. The feed for the flavomycin group was prepared using 4 g premixed food containing 10% flavomycin, which was blended with 100 kg of feed, to reach a concentration of 4 mg/kg. For the *Bacillus* group, 50 mL of *B. subtilis yb-*114,246 fermentation liquid was measured separately and first blended with 5 kg of feed, and then with 95 kg of mass feed. The SEBS feed was prepared by blending 1000 mL of SEBS fermentation liquid with 100 kg of feed. After preparing the five different feedstuffs, the population of *B. subtilis yb-*114,246 was counted using the plate method with a yeast extract peptone dextrose medium. The concentration of Se in all feed types was also measured. The results are listed in Table 2.

Table 1
Nutrient analysis of the basic diet for chicks.

	Ingredient	Starter (0–21) %	Finisher (21–42) %
Item	Corn	58.12	61.75
	Soybean meal	29.15	26.45
	Fish powder	5.00	3.51
	Soybean oil	2.00	3.00
	Premix∗	5.00 [a]	5.00 [a]
	Dicalcium phosphorus	0.47	0.29
	Limestone	0.26	0
Calculated nutrient	Metabolizable energy (MJ /kg)	12.02	12.49
	CP	21	17.5
	Calcium	1	0.85
	Total phosphorus	0.68	0.65
	Available phosphorus	0. 5	0.42
	Lys	1.2	1.0
	Met	0.46	0.32

Note: The premix provides,

[a] vitamins and trace elements per kg diet: Vitamin A (retinyl acetate) 9, 875 IU, Vitamin D_3 (cholecalciferol) 3, 000 IU, Vitamin E (DL-α-tocopheryl acetate) 20 IU, menadione 3.25 mg, Vitamin B_{12} (cyanocobalamin) 0.025 mg, thiamin 1.5 mg, riboflavin 5.0 mg, biotin 0.032mg, folacin 1.25 mg, niacin 12 mg, pantothenic acid 12 mg, and pyridoxine 3.75 mg, manganese 100 mg, zinc 80 mg, iron 80 mg, copper 8 mg, iodine 0.15 mg, and selenium 0.15 mg.

Table 2
Concentration of Se and number of *B. suthilis* in Groups.

Groups	Concentration of Se (ng/g)	number of *B. suthilis*:*B.licheniformis* (CFU/g)
Control (C1)	102.0	0
IS (C2)	602.0	0
BS (C3)	102.0	4.0×10^6
SECB (C4)	602.0	4.0×10^6
Flavomycin (C5)	102	0

2.4. Performance and sample collection

Chicks in every replicate of each treatment group were weighed on days 0 d and 42. Daily feed consumption was accurately recorded. After 42 d, 2 chickens with an average body weight in each replicate were selected (n = 5×2), fasted for 12 h, and then the tissue were harvested under general halothane anesthesia. Tissues of ileum were removed under aseptic conditions, stored in sterile plastic tubes in boxes packed with ice, and immediately transported to our laboratory for quantification of assays.

2.5. Fluorescence in situ hybridization (FISH) assay

The strain of bacteria *B. subtilis* residing in the GIT were investigated using FISH. The probe was designed based on the 16S ribosomal ribonucleic acid of *B. subtilis* yb-1114246 [21]. The length of probe was designed long to ensure the specific binding. Ileal mucosal samples (0.3 g) were fixed by immersion in 10% formaldehyde for 24 h. A 50-μL volume of homogeneous tissue liquid was transferred to poly-L-lysine-coated slides, which were then air-dried on a sterile benchtop for 3 h. The tissue was incubated with lysozyme at 32 °C for 10 min; the slide was then washed with distilled water and immersed in 70% ethanol for 2 min, followed by air drying. Probes with carboxytetramethylrhodamine was designed and conjugated with *B. subtilis yb-1*14,246. The *B. subtilis yb-1*14,246 genetic probe was long to ensure specific integration with samples. Sequence of probe was listed in Table 3. The probe was diluted to 60 nM, denatured at 95 °C for 5 min, and maintained at 4 °C before use. A probe (12 μL) was added to the tissue, followed by incubation at 46 °C for 12 h, and washed with phosphate buffer solution (pH 7.4). The tissue was stained with 4',6-diamidino-2-phenylindole for 5 min, then washed three times with distilled water for 5 min each. After drying, the slides were mounted with fluoromount-GTM (Abcam, Cambridge, UK) and observed with a fluorescence microscope (BX53; Olympus, Tokyo, Japan).

Table 3
The probes *B. subtilis yb-1*14,246.

Bacteria	Genetic sequences 5'-3'
B. subtilis	CGCGATGTAGAGACTGATCGGCCACAATGGAACTGAGACACGGTCCATACTCCTACGTGAGGCTGCAGTAGGGAATC
	TTCCACAATGGTGCTCAAGCCTGATGCGAGCAACACCGCGTGAGTGAGAGAAGGGTTCGGCTCGTAAAGCT
	CTGTGTGTTGGAGAAGAACGTGGTGAGAGTAACTGTTCAGCAGTGACGGTATCCAGACCAGAAAGTCACGGCTAACTTACGTGCCAGCAGCCGCGG

2.6. Quantitative real-time polymerase chain reaction for colonization of B. subtilis

After fermentation in beef extract peptone medium, a tenfold dilution series of *B. subtilis yb-1*14,246 was plated. Colonies of *B. subtilis yb-1*14,246 were counted using the plate method under a microscope to obtain samples of 1 × 104, 105, and 106. Total RNA in each dilution was extracted using the RNA Extraction Kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using a GoScript Reverse System (Invitrogen). First-strand cDNA was synthesized by incubating a reaction mixture containing 11 μL RNA and 1 μL RNase-free dH2O at 70 °C for 3 min, followed by 0 °C for 5 min. A dNTP mixture (1 μL; 10 mmol/L), 4 μL GoScript 5X reaction buffer, 1 μL GoScript reverse transcriptase, 1.5 μL Mg^{2+} (25 mM), and 0.5 μL RNase inhibitor were combined in a total volume of 20 μL and incubated at a 37 °C in a water bath. Primers were designed according to the 16S rRNA of *B. subtilis* KT260179 and are described in Table 4. Amplification was performed in a 20-μL mixture containing 10 μL of 2 × qPCR SYBR Premix Ex-Taq, 2 μL template cDNA, 0.5 μL each primer (10 μmol/L), and 7 μL PCR-grade water. The cycling protocol was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and one cycle for melting curve analysis, consisting of 95 °C for 60 s, 65 °C for 60 s, and 95 °C for 1 s. The amplification curve was generated based on the dilution of the standard curve of *B. subtilis yb-1*14,246. The standard curve of *B. subtilis yb-1*14,246 was described according to the results of qPCR.

Table 4
All of the PCR primers

Gene name	Forward	Reverse
B. subtilis	ACATCCTCGAAGATACAGTGAGA	GCATGACAACTACCACGACCT
TNF-α	CCACAGCTCCGCTCAGAAC	GAGAGGACGATGCCACGAC
IFN-γ	AACCTTCCTGATGGCGTGAA	AAACTCGGAGGATCCACCAG

Samples (0.2 g) of mucous membrane from the distal segment of the ileum were prepared to extract total RNA and qPCR was carried out as described above to evaluate colonization of *B. subtilis*.

2.7. Gut bacterial 16S rDNA sequence and analysis

Samples (0.25 g) of the ileal mucous membrane were prepared (n = 5□10). Microbial DNA was extracted. Final DNA concentration and purity were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA quality was determined using 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers *338F* (5'-ACTCCTACGGGAGGCAGCAG-3') and *806R* (5'-GGACTACHVGGGTWTCTAAT-3') using a thermocycler PCR system (GeneAmp 9700, Applied biosystems, Foster City, CA, USA). PCR was conducted as follows: 3 min of denaturation at 95 °C, 27 cycles: 30 s at 95 °C, 30 s of annealing at 55 °C, 45 s of elongation at 72 °C, and a final extension at 72 °C for 10 min. PCR was performed in triplicate in 20-μL mixtures containing 4 μL of 5×FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Madison, WI, USA) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar concentrations and paired-end sequencing was performed (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to standard protocols. The Illumina sequencing raw data have been deposited into the Sequence Read Archive database (SRP) of NCBI (SRR13290974). The BioProject accession number is PRJNA684959.

2.8. qPCR assays for chicken ileal immune cytokines

Chicken mucosal tissues, collected from the distal segment of the ileum, were washed with ice-cold PBS to remove intestinal contents, and longitudinally cut into small specimens. Chicken mucosal cells were isolated using a PBS buffer containing 1 mM EDTA, 1 mM dithiothreitol, and 5% fetal bovine serum, with shaking at 37 °C for 10 min. Samples of cells were prepared to extract total RNA to evaluate the level of the immune cytokines of tumor necrosis factor-α (TNF-α) and interferon-β (IFN-β) post-Se, *B. subtilis yb-1*14,246, SEBS and flavomycin supplementation *in vivo*. Relative expression levels of target genes were quantitatively normalized against the expression of GAPDH using the ΔΔCT method. Primers for TNF-α and IFN-β were designed according to the chicken RNA genes submitted to NCBI. All PCR primers used in this study are described in Table 4.

2.9. Statistical analyses

In order to facilitate statistical analysis, the names of control, SS, BS, SEBS and flavomycin groups were instead by C1, C2, C3, C4, and C5 respectively in all figures and tables. Body weight, Se concentration, qRT-PCR, and DNA sequencing data were subjected to one-way ANOVA using the GLM procedure of SPSS, with significance reported at $P < 0.05$. Means were further separated using Duncan's multiple range test. All data were statistically processed as repeated measures to determine the interaction of Se and *B. subtilis*. A *P*-value of less than 0.05 was considered statistically significant.

Diversity metrics were calculated using the core-diversity plugin within QIIME2. Feature level alpha diversity indices and operational taxonomic units (OTUs) were used to estimate the microbial diversity within an individual sample. Beta diversity distance measurements were performed with weighted UniFrac to investigate the structural variation in the microbial communities across samples, and then visualized via principal coordinate analysis (PCoA). Co-occurrence analysis between mRNA of immune cytokines of TNF-α, IFN-β and species of bacteria in ileal mucous membrane was performed by calculating Spearman's rank correlations and the network plot. Additionally, the potential KEGG Ortholog (KO) functional profiles of microbial communities were predicted using PICRUSt.

3. Results

3.1. Analysis of SEBS

SEBS was harvested after fermentation of *B. subtilis yb-1*14,246 in medium containing sodium selenite. The medium appeared pale pink in color. The concentration of ionic Se in fermentation supernatant and precipitate of SEBS was measured using HG-AAS, and the concentrations reached 1.77 μg/mL and 48.13 μg/mL, respectively. In the precipitate, Se existed primarily as Se protein (valence 2-) and nanoparticles of Se (valence 0) in the cells of *B. subtilis yb-1*14,246. The live cell of *B. subtilis yb-1*14,246 and SEBS both reached 9.2 · 108 CFU/mL. The appearance and internal structure did not vary between *B. subtilis yb-1*14,246 and SEBS, as proved by SEM and TEM.

3.2. Impact on Growth Performance and Mortality

To compare the probiotic effect of *B. subtilis yb-1*14,246 and SEBS, the measurements regarding body weight of chicks and the results of growth performance and mortality of broiler chickens are shown in Fig. 1. The final body weight of chicks with SEBS supplementation was significantly increased ($P < 0.01$) compared to those of the control and inorganic Se supplemented groups, with the body weight increase being 303 g (Fig. 1a). The mortality of chicks with *B. subtilis yb-1*14,246, SEBS, and flavomycin supplementation was significantly reduced (Fig. 1b, $P < 0.01$). The mortality under SEBS supplementation was the lowest, with a decrease of 3.87 compared to that in the controls ($P < 0.01$).

3.3. Colonization and Levels of B. subtilis yb-114,246 and SEBS in Intestine

To explore the colonization of *B. subtilis yb-1*14,246 in intestinal mucous membranes, we designed an *in vivo* study and employed FISH and qRT-PCR. *B. subtilis yb-1*14,246 is indicated by green spots detected in the distal segment of the ileum by the FISH assay (Fig. 2a) for both *B. subtilis yb-1*14,246 and SEBS supplementation. The qRT-PCR assay results were consistent with the FISH results. The standard curve of *B. subtilis yb-1*14,246 was based on the tenfold dilution series of the fermented culture (Fig. 2b). *B. subtilis yb-1*14,246 growth increased in the distal segment of the ileum (Fig. 2c, $P < 0.01$).

3.4. SEBS Optimized Ileal Microbiota

In order to evaluate the changes of bacterial composition in ileal mucous membranes caused by *B. subtilis yb-1*14,246 colonization, the next generation of sequencing technology was employed. High-throughput sequencing of all samples produced a total of 602,704 clean tags, which were identified as a total of 551 OTUs in all samples showed in Fig. 3. This sequencing depth closely reflects the total microbial species richness. The number of OTUs in control, IS, BS, SEBS and flavomycin was 317, 343, 400, 432, 340 respectively. Chicks with SEBS supplementation is the highest number. Bacterial composition in all supplementary groups was improved compared with control. All five groups are represented by 234 OTUs, contributing 42.47% of the total proportion. Bacterial composition in the ileum showed few differences with SEBS and BS supplementation through the alpha diversity (Shannon index) of mucous bacterial composition both in phylum and genus levels (Fig. 3b, Fig. 3c). The similarities of the weighted UniFrac-based PCoA indicated that the main factors caused 67.68% of the variations (Fig. 3d, R = 0.2274), which influenced the composition of the microbiota. Results of bacterial community structure indicated that the numbers of three specific phyla, namely *Bacteroidetes*, *Actinobacteria*, and *Epsilonbacteraeota*, significantly increased with *B. subtilis yb-1*14,246 and SEBS supplementation compared with those in controls, with the numbers of *Actinobacteria* being higher than those in the IS and flavomycin groups (Fig. 3e).

At the genus level, the bacterial diversity of all supplementation groups was more abundant than that of the control (Fig. 3c, Fig. 3f). Six genera, namely *Candidatus Arthromitus*, *Romboutsia*, *Escherichia-Shigella*, *Enterococcus*, *Gallibacterium*, and *Tyzzerellawere*, represented 87.28% of the total proportion, with *Candidatus Arthromitus* representing 35.54% in controls (Fig. 3f). Further, the genera *Alistipes*, *Helicobacter*, *Ruminococcaceae*, and *Ruminococcus* were observed in the supplementation groups, and *Lactobacillus* and *Bacteroides* were found in the *B. subtilis yb-1*14,246 and SEBS groups. Similarities of PCoA showed that two main factors influenced the bacterial cluster at the genus level, indicating a ratio of 61.09% (Fig. 3e, R = 0.2545). In the PCoA of bacterial OTUs, the SEBS group samples clustered together more than those of any other group. Results from controls were most scattered. These suggest that the bacterial communities were most stable and optimal with SEBS supplementation.

3.4. SEBS Improves Immunity and Metabolism

The relationship between the microbiota of ileal mucous membranes and chicken body function was assessed through KEGG pathway classification and one-way ANOVA. After sequencing the 16SrDNA of ileal mucous samples, we got the detailed statistical results on the metabolism and digestion of nutritional substances, DNA, RNA and protein expression influenced by bacterial OUT in phylum or genus levels. Some dominate genus of bacteria were chosen to draw the figure on the bacterial composition with functions. Results (Fig. 4a and 4b) indicated that more microbes were found to promote the genetic expression of amino acids, carbohydrates, co-enzymes, lipid transport and metabolism ($P < 0.01$), energy production and conversion ($P < 0.01$), signal transduction mechanisms, and defense mechanisms ($P < 0.05$). Further, primary infectious diseases caused by pathogenic bacteria were analyzed (Fig. 4c), which showed that all four supplements strengthened defenses against bacterial invasion of chicken epithelial cells, compared to controls ($P < 0.01$).

The mainly changed OUT on genus level in ileal mucous membrane was chosen to analyze the differences of pathogen according to the sequencing statistical results. We chose the pathogen covered major proportion and easily caused disease. With *B. subtilis yb-1*14,246, SEBS, and flavomycin supplementation, body defenses against biofilm formation by *Vibrio cholerae* improved significantly ($P < 0.01$). Further, defense against *Salmonella* infection and Pertussis improved in two *B. subtilis yb-1*14,246 and flavomycin supplementation groups. Moreover, chicks receiving SEBS exhibited enhanced defense against pathogenic *Escherichia coli* infection and Shigellosis ($P < 0.01$) (Fig. 4c) according to one-way ANOVA analysis.

TNF-α, IFN-β were two important anti-infection factors in body immunity, which were chosen to evaluate the immune status. Immune cytokines TNF-α, IFN-β in the chicken ileal mucosa were further inspected using qRT-PCR. The mRNA expression of cytokines TNF-α and IFN-β were monitored (Fig. 5), with increased expression of two cytokines observed in in SEBS, BS, and flavomycin groups, compared to controls ($P < 0.01$). In the IS-supplemented group, the expression IFN-β were significantly improved compared with control groups ($P < 0.01$). The expression of cytokines TNF-α and IFN-β in the *B. subtilis yb-1*14,246 group was higher than those observed in the SEBS group ($P < 0.01$).

3.5. Correlation between on bacterial composition and intestinal immune cytokines

Spearman's correlation analysis on species of bacteria and chicken immune factors TNF-α, IFN-β was conducted to unveil the relationship. Results (Fig. 6) indicated that the abundance of species of uncultured *Candidatus arthromitus* were negatively correlated with BD1 concentration and anti infection in ileal mucous membranes ($P < 0.01$), and included *Romboutsia* regarding anti-infection analysis. However, abundant species of unclassified *Lachnospiraceae*, uncultured *Anaerosporobacter*, uncultured *Ruminococcaceae_UCG-014*, uncultured *Peptococcus*, *Lactobacillus salivarius*, and unclassified *Butyricicoccus* were positively correlated with two environmental factors ($P < 0.01$). Species of unclassified *Lachnospiraceae*, uncultured *Anaerosporobacter*, *Ruminococcaceae_UCG-014*, *Peptococcus*, *Lactobacillus salivarius*, and unclassified *Butyricicoccus* were all increased in SEBS-supplemented chicks. *Lachnospiraceaer*, *Ruminococcaceae_UCG-014*, *Peptococcus*, *Lactobacillus salivarius*, and *Butyricicoccus* aid in digestion and nutrient absorption.

4. Discussion

To explore the combined effect of Se and *B. subtilis yb-1*14,246, we first cultured SEBS, then analyzed its morphological and biochemical characteristics. These characteristics did not exhibit any differences compared to *B. subtilis yb-1*14,246 after being bio-transformed with inorganic Se. Selenomethionine was proved as the primary ionic form of Se in bacteria after fermentation [22]. The ionic form of Se in the supernatant and precipitate of fermented SEBS medium constituted nano-Se in red particles [23]. Hence, the composition of Se in the supernatant and precipitate of fermented SEBS medium was mainly selenomethionine and nano-Se in red particles, which confers a pale pink color to the medium. The valences of Se changed from 4^+ to 2^- and 0. Further, broiler chickens were supplemented with SEBS to unveil the effect on intestinal innate immune expression of BD1 and its potential mechanism.

Chicks exhibited higher final body weights in the chicken study with *B. subtilis yb-1*14,246 and SEBS supplementation. The growth-promoting effect was also shown in the final body weight of the *B. subtilis yb-1*14,246, SEBS, and flavomycin groups. This result was consistent with those of previous studies [24, 25]. Both Se and *B. subtilis yb-1*14,246 modulate the growth performance of chickens [26, 27]. However, we did not observe any significant effects of IS supplementation. In our study, we supplemented basal feedstuff with a dose of 0.5 μg/g Se in an inorganic form, which had no positive effects on broiler chickens; this is in accordance with our previous studies [28].

Exploring the colonization of bacteria *in vivo* must be more eloquent than in cells *in vitro.* Previous study reported that the composition of ileum was most abundant among three segments of small intestine, which owing 10^7CFU/g bacteria [29]. *B. subtilis* mostly colonized in the ileal mucous membrane [30]. Our results also proved *B. subtilis yb-1*14,246 fed to chicks can colonize the ileal mucous membranes, and the bacterial number was ascertained using FISH and qRT-PCR assays. The bacteria used nutrients in the intestine for propagation, while conferring health benefits to the chick, indicating a reciprocal relationship [31, 32]. With bacterial growth, metabolites of *B. subtilis yb-1*14,246 include antimicrobial substances and digestive enzymes, such as protease, lipase, and amylase, which play important roles in maintaining health and breaking down feedstuff for nutrient absorption [33]. Chickens receiving SEBS exhibited a higher final body weight and greater feed utilization efficiency than did control chickens, compared with the group receiving *B. subtilis yb-1*14,246, suggesting that this treatment was more efficient in regulating growth for the biological roles of Se than did *B. subtilis yb-1*14,246 alone. SEBS combined the merits of Se and *B. subtilis yb-1*14,246 to improve growth performance of chicks.

Accompanied by the colonization of *B. subtilis yb-1*14,246, immunity was also improved through such reciprocal pathways. The colonization of *B. subtilis yb-1*14,246 in the ileal mucous membrane improved bacterial composition in the phylum and genus levels. The genera *Lactobacillus*, *Peptococcus*, *Butyricicoccus*, and *Ruminococcaceae_UCG-014* exhibited probiotic functions in the body, and the proportion of these bacteria increased in chicks receiving *B. subtilis yb-1*14,246 and SEBS supplementation [34–36]. Meanwhile, the ration of conditioned pathogens or pathogens, such as *Escherichia-Shigella*, *Vibrio cholerae*, *Salmonella*, and *Pertussis bacilli*, significantly declined. Improved immunity can be indirectly attributed to these results. Abundant species of unclassified *Lachnospiraceae*, uncultured *Anaerosporobacter*, uncultured *Ruminococcaceae_UCG-014*, uncultured *Peptococcus*, *Lactobacillus salivarius*, and unclassified *Butyricicoccus* can be detected in *B. subtilis yb-1*14,246 and SEBS groups. The species of *Lachnospiraceae*, *Ruminococcaceae_UCG-014*, *Peptococcus*, *Lactobacillus salivarius*, *Butyricicoccus*, and *Anaerosporobacter* improved overall health [37–40]. KO functional profiles of microbial communities indicated that metabolism and immunity were improved by these species of bacteria. Body immunity was improved by optimized intestinal microbiota.

In addition, supplementary 0.5 ug/mg Se in diet showed improved effects on bacterial composition in ileal membrane of chick. Our results advised that the bacterial abundances in phylum level were increased, with more OTUs in *Actinobacteria*. In further, the abundances in *Lactobacillus*, *Ruminococcaceae*, and *Ruminococcus* were enriched with Se supplementation. These results were also reported in other studies, selenium in nano particles indicated benefits on sone genus of beneficial bacteria such as *Faecalibacterium prausnitzii* and *Lactobacillus* in poultry gut [41, 42]. Se supplementation enriched the bacterial diversity compared to that in controls, which enhanced nutrient metabolism and immunity, as indicated by the results of KEGG function classification. Supplementary suitable dose of Se could help body establish improved immunity and antioxidation and optimized bacterial composition. But it had no significant effect on live weight gain or abundance of potentially pathogenic bacteria [43], which was correlated with our results.

The effect of SEBS was greater than that of IS in this experiment, suggesting that Se availability was greater with SEBS supplementation. Therefore, SEBS combined the activities of se and *B. subtilis yb-1*14,246 played more active roles than single used.

5. Conclusion

Our study reported the colonization of probiotic bacteria *B. subtilis yb-1*14,246 in distal ileal mucous membrane using FISH and qRT-PCR. Thereafter, we observed that the composition of intestinal microbiota and immunity were improved under the action of colonization. Se binding to the body of *B. subtilis yb-1*14,246 can more promote body growth and immunity. Combined use of Se and *B. subtilis yb-1*14,246 as SEBS induced further improvements compared to those observed when administered alone, improved more body growth and better immune status, decreased mortality. Our research provided a new avenue in use of probiotics and essential micro-elements.

Abbreviations

B. subtilis: *Bacillus subtilis*; SEBS: Selenium-enriched *Bacillus subtilis*; GIT: gastrointestinal tract; FISH: fluorescence in situ hybridization; qPCR: quantitative real-time polymerase chain reaction; Se: selenium.

Declarations

Ethics approval and consent to participate

Animal experiments were approved and performed in accordance with the experimental guidelines of the Institutional Animal Care and Use Committee of China. The experimental protocols in this study, including animal husbandry and slaughter, were approved by the Institute of Animal Science and Welfare of Anhui Province (no. IASWAP2017120649).

Consent for publication

Not applicable.

Availability of data and materials

The sequencing data of cecal microbiota is deposited into the Sequence Read Archive database (SRP) of NCBI (SRR13290974). The BioProject accession number is PRJNA684959.

Conflicts interests

The authors declare that there is no conflict of interest.

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Author contributions

YJ and HK conceived the idea of the study. YJ carried out animal experiment, data analysis, and drafted the manuscript. WJ and ZM did the analysis, LQ and XX did the qRT-PCR of bacteria. WJ and ZM took part in samples collection. HK and ZH revised the manuscript provided the fund.

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Figures

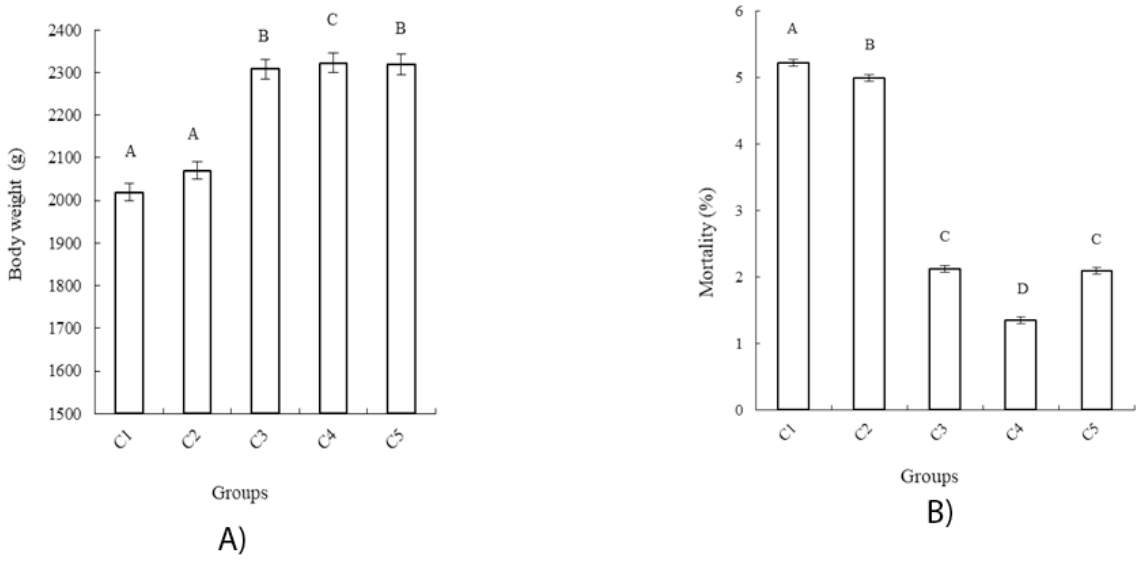

Figure 1

Chicken growth performance and mortality. Chicks were treated with control, or with SS, BS, SEBS and flavomycin supplementation. The names of control, SS, BS, SEBS and flavomycin groups were instead by C1, C2, C3, C4, and C5 respectively. a Growth performance of different groups. b Mortality of different groups. Data was statistically processed as repeated measurements. The different superscript capital letters in the same color of column in the same experimental duration mean significant difference at 0.01 levels ($P<0.01$).

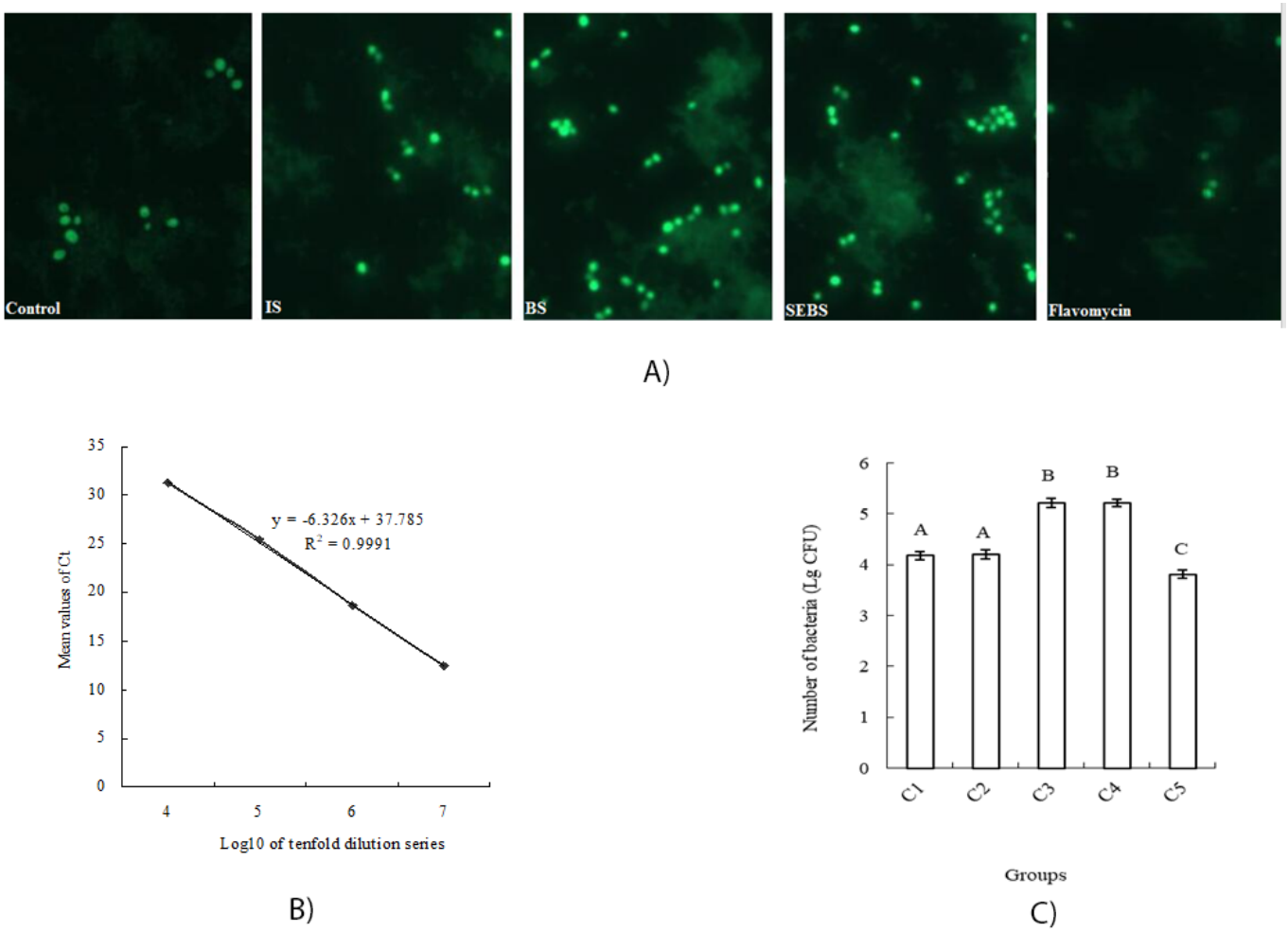

Figure 2

Colonization of B. subtilis yb-114,246 of different groups with FISH and qPCR assays. a Colonization of B. subtilis yb-114,246 in the distal ileum measured with FISH. b The standard curve of B. subtilis yb-114,246 with qPCR assay. c Number of Colonized B. subtilis yb-114,246. Values are means, with standard errors are represented by vertical bars. Mean value was significantly different from that of the control group by one-way ANOVA followed by Tukey's multiple comparison tests: the different superscript capital letters in the same color of column in the same experimental duration mean significant difference at 0.01 levels ($P<0.01$).

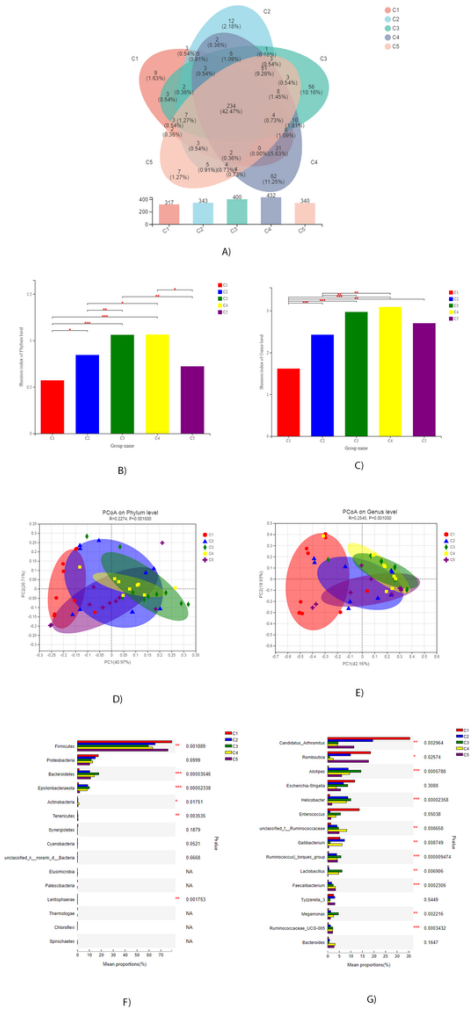

Figure 3

The overall profile of bacterial composition in ileal mucous membrane. a The bacterial OTU in ileal mucous membrane (Venn figures). The number in the color circus or overlapped circus represented the owned OTU in one or more groups and correlated proportion. b Bacterial composition in phylum level. c Bacterial composition in phylum genus level. One-way ANOVA analysis on bacterial composition in phylum level. * in the same column means P < 0.05, ** means P < 0.01, and *** means P < 0.001. d PCoA analysis in genus level. e PCoA analysis in phylum level. PCoA analysis of UniFrac distance metric of bacterial OTUs. The figure was drawn by the ANOSIM of UniFrac distance metric. f Bacterial composition in phylum level. g Bacterial composition in phylum genus level. One-way ANOVA analysis on bacterial composition in phylum level. * in the same column means P < 0.05, ** means P < 0.01, and *** means P < 0.001.

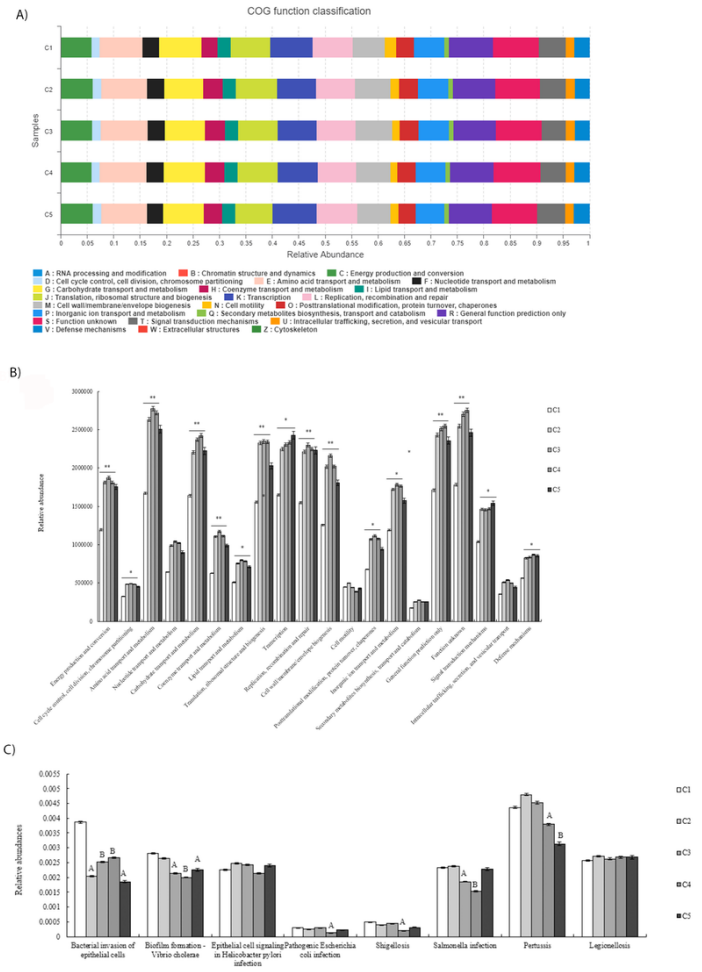

Figure 4

Function classification based on bacterial composition. a KEGG pathway function classification. STAMP software was applied to detect the differentially abundant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among groups with false discovery rate correction. b One-way of ANOVA analysis between bacterial composition and function. * mean significant difference at 0.05 levels (P<0.05), **mean significant difference at 0.01 levels (P<0.01). After sequencing the 16SrDNA of ileal mucous samples, we got the detailed statistical results on the metabolism and digestion of nutritional substances, DNA, RNA and protein expression influenced by bacterial OUT in phylum or genus levels. Some dominate genus of bacteria were chosen to draw the figure on the bacterial composition with functions. c Relative abundance of pathogen in One-way of ANOVA analysis of KEGG pathway function classification caused by pathogenic bacteria. The different superscript capital letters in the same color of column in the same experimental duration mean significant difference at 0.01 levels (P<0.01). The one-way ANOVA analysis was performed by post hoc tests. The mainly changed OUT on genus level in ileal mucous membrane was chosen to analyze the differences of pathogen according to the sequencing statistical results. We chose the pathogen covered major proportion and easily caused disease.

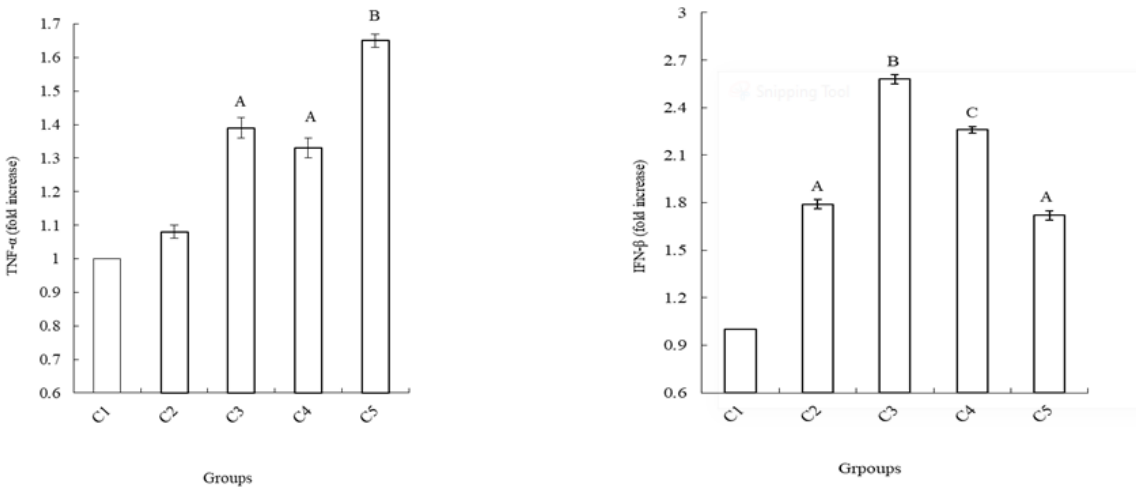

Figure 5

The expression of cytokines intestinal mucous cells. mRNA: glyceraldehyde phosphate dehydrogenase (GAPDH) internal control was used for statistical comparison. Values are means, with standard errors are represented by vertical bars. Mean value was significantly different from that of the control group by one-way ANOVA followed by Tukey's multiple comparison tests: the different superscript capital letters in the same color of column in the same experimental duration mean significant difference at 0.01 levels (P<0.01).

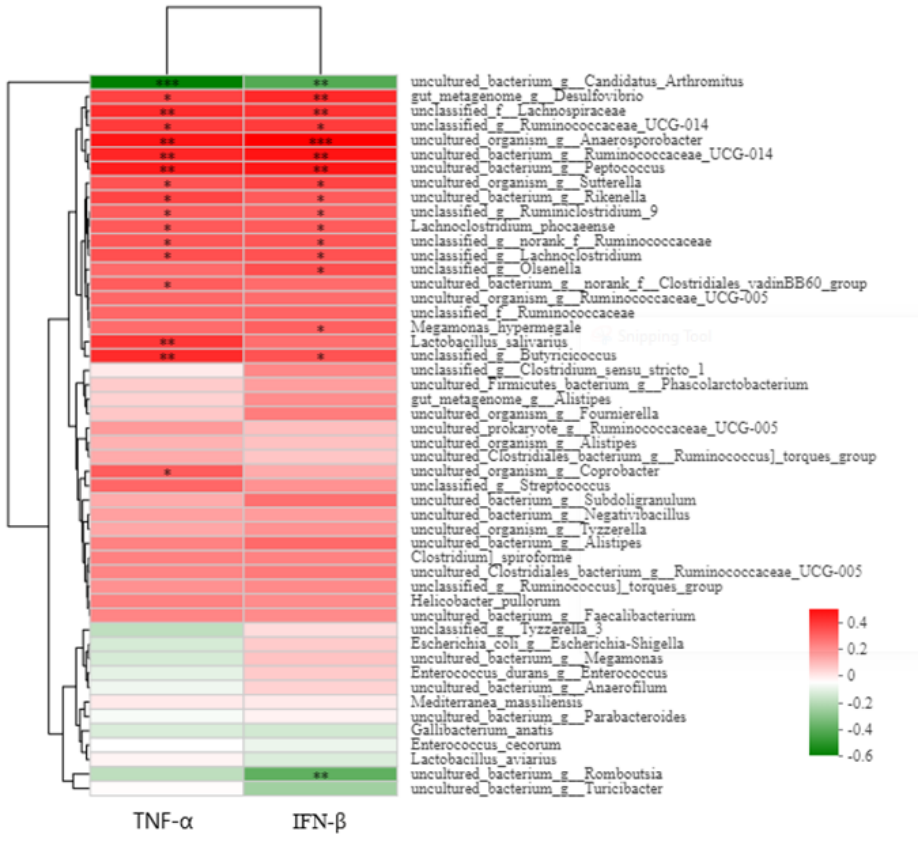

Figure 6

The Spearman's correlation analysis on mRNA of immune cytokines of TNF-α, IFN-β and species of bacteria in ileal mucous membrane. * mean significant difference at 0.05 levels (P<0.05), **mean significant difference at 0.01 levels (P<0.01), *** mean significant difference at 0.001 levels (P < 0.001). Relative abundance is indicated by a color gradient from green to red, with green representing low abundance and red representing high abundance.